In vivo suppression of osteosarcoma pulmonary metastasis with intravenous osteocalcin promoter-based toxic gene therapy

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Pulmonary metastases are the main cause of death of patients with several types of cancer, including osteosarcoma, renal cell carcinoma, malignant melanoma, and breast cancer. Previously, we demonstrated that intralesional injection of the recombinant adenovirus (Ad) vector containing the herpes simplex virus thymidine kinase (TK) gene driven by an osteocalcin (OC) promoter (Ad-OC-TK) effectively suppressed the growth of osteosarcoma cells *in vitro* and tumors *in vivo* in a tumor-specific manner when supplemented with the prodrug acyclovir (ACV). In this communication, we studied the potential efficacy of the treatment of osteosarcoma pulmonary metastases with a systemic delivery route of Ad-OC-TK supplemented with ACV. We established osteosarcoma lung metastases in nude mice by the intravenous injection of rat osteosarcoma cells, ROS 17/2.8. These cells colonized and formed tumor nodules within 1 week in the lungs of nude mice. Whereas systemic delivery of a recombinant Ad vector containing the *Escherichia coli* β-galactosidase (β-gal) gene driven by a Rous sarcoma virus universal promoter (Ad-RSV-β-gal) resulted in the nonspecific expression of β-gal activity in the lung parenchyma, Ad-OC-β-gal administration resulted in specific β-gal expression in tumor cells deposited in the lung. When nude mice bearing ROS 17/2.8 lung tumors were treated with systemic Ad-OC-TK through tail vein administration, subsequent intraperitoneal ACV treatment significantly decreased the number of tumor nodules ($P < .0001$) and the net lung wet weight ($P = .0005$) while significantly increasing ($.005 < P < .01$) the survival of animals, when compared with untreated and Ad-OC-TK- or ACV-treated control groups. These results suggest that Ad-OC-TK/ACV may be used as a systemic therapy for the treatment of osteosarcoma lung metastasis.

Key words: Osteocalcin promoter; toxic gene therapy; pulmonary metastasis; osteosarcoma; adenovirus; thymidine kinase.

Toxic gene therapy for the treatment of cancer continues to gain prominence in basic research; however, it remains limited in clinical application because of an inability to deliver the toxic gene to the tumor cells with specificity. Many vectors (eg, retroviruses, retroviral-producing cells, adenoviruses (Ads), liposomes, and others) can deliver therapeutic or toxic genes to target cells. Both localized delivery and restricted gene expression to the primary tumor have been accomplished via direct injection of therapeutic viruses in animal models[1-4] and clinical trials.[5,6] However, this approach is not feasible for the treatment of metastatic disease because of the presence of multiple lesions that would each require separate injection and manipulation. Therefore, alternative approaches for the treatment of metastatic disease with gene therapy must be developed.

Systemic delivery of therapeutic genes is attractive for targeting metastatic disease in general and pulmonary metastases in particular. Because the pulmonary vascular system would be encountered first, the Ad would be trapped in the lung parenchyma, allowing for higher infectivity. Lesoon-Wood et al[7] reported that the systemic delivery of wild-type p53 complexed with liposomes targeted the p53 mutated breast cancer cell line (MDA-MB435), inhibited primary tumor growth by 60%, and decreased pulmonary metastases in nude mice. Vile et al[8] demonstrated the inhibition of B-16 melanoma pulmonary metastases in syngeneic immunocompetent mice by the systemic delivery of retrovirus, using a tyrosinase promoter to drive the expression of the toxic thymidine kinase (TK) gene.

Compared with liposomes or a retrovirus, an Ad has several advantages in a systemic delivery strategy, such as its high infectivity *in vivo* and production techniques

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that can achieve high viral titers. However, Brand et al[9] recently reported that the systemic administration of Ad-containing TK under the control of a cytomegalovirus universal promoter supplemented with ganciclovir treatment induced severe hepatotoxic effects. This study suggested that restricting toxic gene (TK) expression with a tissue-specific promoter may be necessary prior to the consideration of systemic Ad vector delivery. Moreover, for more effective treatment of metastatic diseases, the tissue-specific promoter should limit the toxic gene expression in normal tissues so that Ad containing tissue-specific promoters can be applied in higher doses than those containing universal promoter-based toxic gene therapy.

Osteosarcoma is an attractive model for the study of the potential therapeutic efficacy of systemic cancer gene therapy for the treatment of pulmonary metastases because a significant number of osteosarcoma patients eventually develop lung metastasis. In terms of initial therapy, surgical resection of the primary lesion and adjunctive chemotherapy are the current mainstays. For the 20% of osteosarcoma patients that present with metastatic disease, 80% will require additional therapy for relapse. Of the 80% that present with local disease, 35% will require additional therapy for relapse after surgery and adjunctive chemotherapy.[10] Therefore, 44% of patients diagnosed with osteosarcoma will fail conventional first line therapy. Patients developing recurrent disease usually have a poor prognosis, dying within 1 year of the development of metastatic disease.[11–14] New therapeutic approaches that can be applied either separately or in conjunction with current modalities to treat osteosarcoma pulmonary metastases are needed.

The osteocalcin (OC) promoter has been shown to be highly effective in directing the transcription of reporter genes in both rat and human osteosarcoma cell lines.[4,15] Recently, we reported that a recombinant Ad (rAd) containing the TK gene under the control of the OC promoter, when supplemented with a prodrug acyclovir (ACV), could suppress osteosarcoma growth through intralesional injection in both rat and human osteosarcoma models.[1,4] In this report, we extended our study of the use of the tissue-specific promoter for toxic gene therapy to the treatment of pulmonary metastasis by the systemic delivery of Ad. We demonstrated the suppression of osteosarcoma pulmonary metastases with systemically delivered Ad-OC-TK followed by ACV in an athymic nude mouse model. This study illustrated the potential use of a tumor-specific promoter for toxic gene therapy through a systemic delivery strategy.

MATERIALS AND METHODS

Cells and cell culture

ROS 17/2.8, a rat osteoblastic osteosarcoma cell line, was generously provided by Dr. Cindy Farrach-Carson (The University of Texas Dental Branch, Houston, Tex). ROS 17/2.8 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with penicillin (100 U/mL), streptomycin (100 mg/mL), and 10% fetal bovine serum (Sigma, St. Louis, Mo). The cells were fed three times per week with fresh growth media.

Construction and preparation of rAd vectors

The construction of a rAd vector containing OC promoter TK (Ad-OC-TK) completed as previously described.[4] The rAd vectors containing either OC promoter β-galactosidase (β-gal) (Ad-OC-β-gal) or RSV (Rous sarcoma virus) promoter β-gal (Ad-RSV-β-gal) were constructed in similar fashion using the same protocol. Briefly, an OC or RSV promoter plus the *Escherichia coli* β-gal (*lacZ*) gene and the polyadenylation signal from simian virus 40 were initially cloned into a plasmid pΔE1sp1A (a gift of Dr. Frank Graham, McMaster University, Hamilton, Ontario, Canada) to generate the shuttle vectors pΔE1sp1A-OC-β-gal or pΔE1sp1A-RSV-β-gal, respectively. The replication-defective Ads Ad-OC-β-gal and Ad-RSV-β-gal were produced by cotransfecting pΔE1sp1A-OC-β-gal or pΔE1sp1A-RSV-β-gal with a rAd vector, pJM17, into 293 cells using a 1,2-dioleoyloxy-3-trimethylammonium propane-mediated (Boehringer Mannheim, Indianapolis, Ind) transfection method.[16] The cell lysates were prepared from dishes that showed the cytopathic effect of Ad infection. A polymerase chain reaction analysis was performed to identify both rAds and wild-type Ads.[16] rAds were propagated in 293 cells and purified by the CsCl centrifugation method.[17] The purified virus stock was then dialyzed against 10 mM tris(hydroxymethyl)aminomethane buffer (pH 7.5) containing 1 mM $MgCl_2$ and 10% glycerol. The plaque-forming units (PFUs) of the viruses were measured by a standard biologic plaque-forming assay and optical density measurements.[17]

Animal model of osteosarcoma pulmonary metastasis

Athymic BALB/c (nu/nu) mice that were 5 to 6 weeks old were purchased from Harlan Sprague-Dawley (Houston, Tex). A tail vein injection of 5×10^5 ROS 17/2.8 cells in 50 μL of culture medium resulted in 100% histological incidence of pulmonary metastasis at 7 days ($n = 8$, data not shown). All mice were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care, and all animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Immunohistochemical staining

Removed lung tissues were fixed in 4% buffered formaldehyde, embedded in a paraffin block, and then sectioned. Tissue sections were deparaffinized, treated with 3% hydrogen peroxide (H_2O_2) and blocked with Super Block (Scytek Laboratories, Logan, Utah). To detect the expression of *E. coli* β-gal, tissue sections previously blocked with Super Block were incubated with either 1:1000 diluted rabbit anti-*E. coli* β-gal polyclonal antibodies (Abs) (5 prime–3 prime, Inc., Boulder, Colo) or control rabbit serum at 4°C for 24 hours. Tissue sections were washed thoroughly and incubated for 1 hour with biotinylated goat anti-immunoglobulin Ab (Multilink, BioGenex, San Ramon, Calif) followed by a 1-hour incubation with peroxidase-conjugated streptavidin (BioGenex Laboratories, San Ramo, Calif) at room temperature. Finally, the tissue sections were incubated with the substrate 3-amino-9-ethylcarbazole for color development.

Treatment of pulmonary metastasis with Ad-OC-TK

The osteosarcoma pulmonary metastasis model was established as described above. Ad-OC-TK (5×10^8 PFUs per 50

μL) was injected twice via tail vein at days 7 and 14 after ROS 17/2.8 injection. Daily intraperitoneal ACV administration (40 mg/kg body weight) started at day 6 after ROS 17/2.8 tumor cell inoculation and continued for 15 days. All animals were sacrificed and examined on day 25. The lungs were removed, photographed, and weighed, and the metastatic deposits were observed under a low-powered (×20) stereo microscope. Histomorphologic observations were made for all specimens according to a standard procedure. The Student *t* test was employed to analyze the statistical significance of differences among control and treatment groups.

Long-term survival study

The end-points of the long-term survival study were either animal death or sacrifice upon request by animal care takers because of excessive tumor burden causing animal distress, lethargy, or weight loss. The survival rate of the animals was analyzed by the Kaplan-Meier survival curve. The statistical significance of this study was analyzed with the generalized Wilcoxon signed-rank test.[18]

RESULTS

Establishment of osteosarcoma lung metastasis

The ability of ROS 17/2.8 cells to colonize and form tumors in the lungs of nude mice was tested by injecting cells directly into the tail vein. Two inoculating cell numbers, 5×10^5 and 1×10^6 cells, were selected for intravenous (i.v.) administration into mice via tail vein. Tumor nodules were found in the lungs of all animals within 1 week (four animals per group). Lung metastases appeared to be specific because no other organs were found to harbor gross tumor mass upon complete necropsy. An additional 46 animals studied subsequently demonstrated pulmonary metastasis upon exploration at various time points beyond 1 week.

Specific targeting of osteosarcoma lung metastasis with i.v. OC promoter-driven gene therapy

To test whether the OC promoter can mediate gene expression in normal lung cells, we constructed Ad-OC-β-gal and Ad-RSV-β-gal to have transcriptional control of β-gal gene expression under the OC promoter and the RSV universal promoter, respectively. Ad-OC-β-gal (1×10^9 PFUs per 50 μL), Ad-RSV-β-gal (1×10^9 PFUs per 50 μL), or 50 μL of phosphate-buffered saline (PBS) as a control was injected into mice 7 days after ROS 17/2.8 cell injection via the tail vein. Mice were sacrificed, and lungs were removed for additional analysis 48 hours after a single tail vein injection. The expression of β-gal in the lung tissue and tumor nodules was detected by immunohistochemical staining with anti-*E. coli* β-gal Ab.

In the Ad-RSV-β-gal-treated mice, anti-β-gal immunoreactivity was observed in both osteosarcoma lung metastases and normal lung tissue (Fig 1c). Conversely, in the Ad-OC-β-gal-treated mice, anti-β-gal immunoreactivity was detected primarily in osteosarcoma lung metastases and not in normal lung tissue (Fig 1d). No anti-β-gal immunoreactivity was observed in the PBS-treated host (Fig 1b). These results demonstrate that OC promoter-mediated gene expression is localized preferentially in osteosarcoma tumors deposited in the lung but is not localized in normal lung tissues.

Suppression of osteosarcoma lung metastasis with i.v. Ad-OC-TK gene therapy

We subsequently tested the therapeutic efficacy of i.v. Ad-OC-TK gene therapy for the treatment of osteosarcoma lung metastases. We treated 20 mice bearing ROS 17/2.8 tumor lung metastases with either PBS (control), Ad-OC-TK alone, ACV alone, or Ad-OC-TK/ACV. Animals were sacrificed and analyzed on day 25 after ROS 17/2.8 inoculation. Tumor nodules on the lung surface were counted with stereo-optic magnification, the lung wet weights were measured, and all the lung specimens were subjected to histological analysis.

Gross metastatic lung nodules were observed on the lung surfaces in all of the animals (Fig 2, a–d). In the Ad-OC-TK/ACV-treated group, both the number of nodules ($P < .0001$) and the lung wet weight ($P = .0005$) were significantly lower than in the PBS-treated control group (Figs 3 and 4). Histologically, osteosarcoma pulmonary metastases were identified in the lung tissue of all 20 animals. No statistical difference was demonstrated in either the number or the size of pulmonary metastases in the PBS, Ad-OC-TK alone, or ACV alone control groups. In comparison with PBS-treated specimens (Fig 5a), the tumors in animals treated with Ad-OC-TK and ACV had a marked decrease in tumor size and demonstrated extensive necrosis (see Fig 5b, arrows).

Prolonged survival with Ad-OC-TK gene therapy

As described above, 26 mice were inoculated with ROS 17/2.8 tumor cells. Animals received either PBS, Ad-OC-TK alone, ACV alone, or Ad-OC-TK/ACV, and survivals were compared. No obvious difference in survival was detected among the three control groups: PBS, Ad-OC-TK alone, or ACV alone. The survival of the Ad-OC-TK/ACV-treated group, however, was significantly prolonged ($.005 < P < .01$, generalized Wilcoxon signed-rank test). The mean survival time was 27.1 ± 2.9 days for the control animals and 36.6 ± 5.8 days for the Ad-OC-TK/ACV-treated animals (Fig 6).

DISCUSSION

Since the lung epithelium contains the first capillary bed encountered by therapeutic agents administered systemically, several investigators have explored the use of a venous system to deliver therapeutic genes to the lung by cationic liposomes[7,19–21] or retroviral vectors.[8] We have explored a new treatment strategy to target pulmonary metastases using a tumor-specific OC promoter-based toxic gene therapy administered via a systemic route. Recently, we have shown that the intratumoral injection of Ad-OC-TK to both human and rat osteosarcoma tumors grown at subcutaneous sites significantly im-

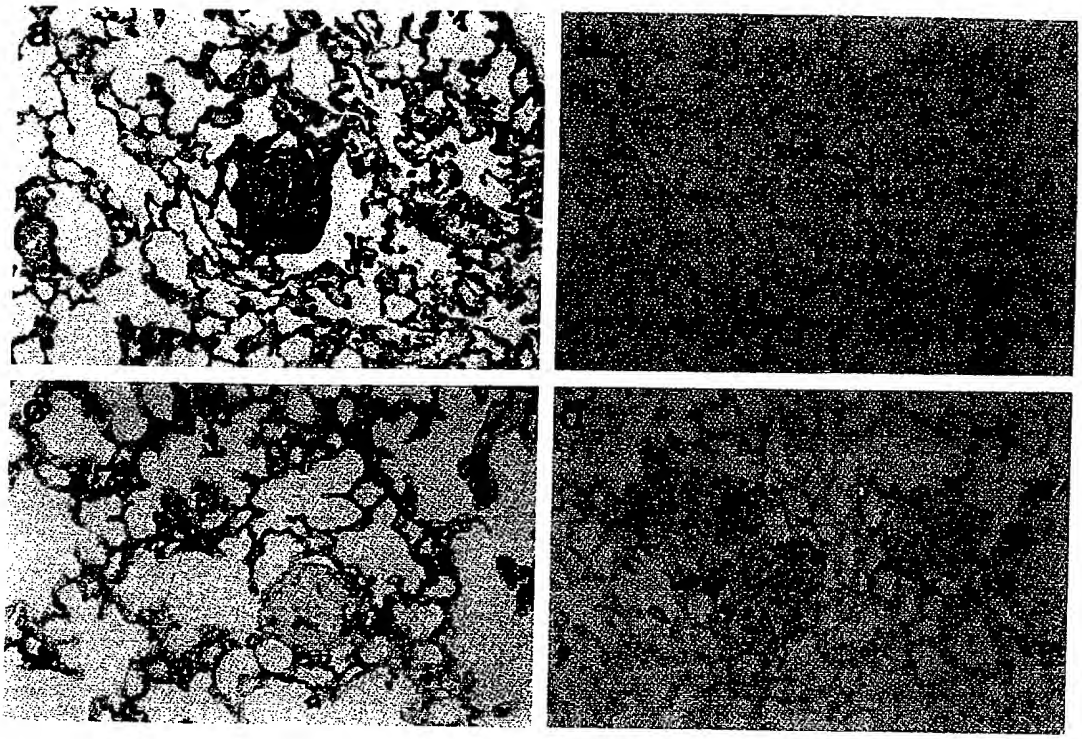

Figure 1. Tissue-specific targeting of osteosarcoma lung metastasis with Ad-OC-β-gal. PBS (50 μL), Ad-OC-β-gal (1 × 10^9 PFUs per 50 μL), or Ad-RSV-β-gal (1 × 10^9 PFUs per 50 μL) was injected into nude mice bearing osteosarcoma lung metastases via the tail vein to study the tissue-specific activity of the OC promoter in a lung environment. Animals were sacrificed 2 days after virus inoculation. Lungs were removed and processed for hematoxylin and eosin staining (a) or for immunohistostaining with anti-β-gal Ab (b, c, and d). Hematoxylin and eosin staining showed the presence of osteosarcoma lung metastasis **(a)**. Control animals received PBS alone and did not show brown positive staining with anti-β-gal Ab **(b)**. Animals receiving Ad-RSV-β-gal expressed β-gal in both lung tissue and tumor nodules **(c)**. Animals receiving Ad-OC-β-gal expressed β-gal only in tumors but not in normal lung tissue **(d)**. All tissue sections were photographed at high power.

paired tumor growth *in vitro* and *in vivo*.[4] Moreover, we noted that the combined administration of Ad-OC-TK/ACV plus methotrexate further improved the therapeutic efficacy of gene therapy for osteosarcoma cell growth both *in vitro* and *in vivo*.[1] Since osteosarcoma primarily metastasizes in the lung, and lung vasculature is considered to be the first major capillary bed that a systemically administered therapeutic agent encounters, we designed a strategy to target osteosarcoma pulmonary metastasis by the administration of Ad-OC-TK/ACV in an animal model. β-gal reporter gene expression under the transcriptional control of the OC promoter is specifically expressed in osteosarcoma cells rather than normal lung parenchyma. In comparison with control animals, systemically delivered Ad-OC-TK/ACV (via an i.v. route) significantly retarded the growth of osteosarcoma pulmonary metastases and improved the survival of treated animals.

Figure 2. Therapeutic effect of Ad-OC-TK/ACV. Animals bearing osteosarcoma lung metastases were treated with Ad-OC-TK/ACV **(a)**, PBS **(b)**, Ad-OC-TK **(c)**, or ACV **(d)** as described above. Animals were sacrificed 25 days after tumor cell inoculation, and lungs were removed for analysis. Note that fewer pulmonary metastatic nodules were found on the lung surface of animals with Ad-OC-TK/ACV treatment (a) than on the lung surface of control animals receiving other treatments (b, c, and d).

While a limited number of tumor cells in the lung may be infected by Ad-OC-TK, as judged by the immunostaining of a comparable virus, Ad-OC-β-gal (Fig 1d), a surprisingly potent growth-inhibiting effect by Ad-OC-TK/ACV was noted in osteosarcoma lung metastases. This biologic effect is most likely derived from the existence of close gap junctions between osteosarcoma cells,[22] which allow the phosphorylated form of ACV to exert its full bystander effect.

The observation that Ad-OC-TK/ACV effectively inhibited the growth of osteosarcoma lung metastases when delivered by an intravascular route raises the question of how to deliver therapeutic viruses via local regional perfusion. For example, employing a Swan-Ganz-type catheter may achieve improved local delivery. The isolated single-lung perfusion technique for the chemotherapy of lung metastasis has increased the con-

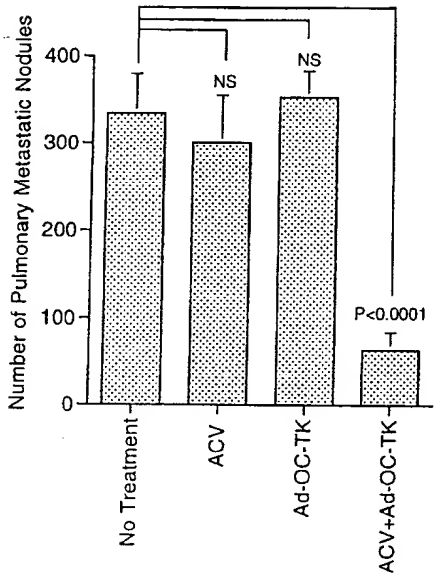

Figure 3. Reduction of the number of pulmonary metastatic nodules with Ad-OC-TK/ACV treatment. Animals bearing osteosarcoma lung metastases were treated with PBS, ACV, Ad-OC-TK, or Ad-OC-TK/ACV as described above. Animals were sacrificed 25 days after tumor cell inoculation, and lungs were removed for analysis. Animals receiving Ad-OC-TK/ACV treatment had significantly fewer lung tumor nodules compared with animals receiving other treatments ($P < .0001$, Student's t test). There were no significant differences in the number of lung tumor nodules between animals receiving PBS, ACV, or Ad-OC-TK treatment.

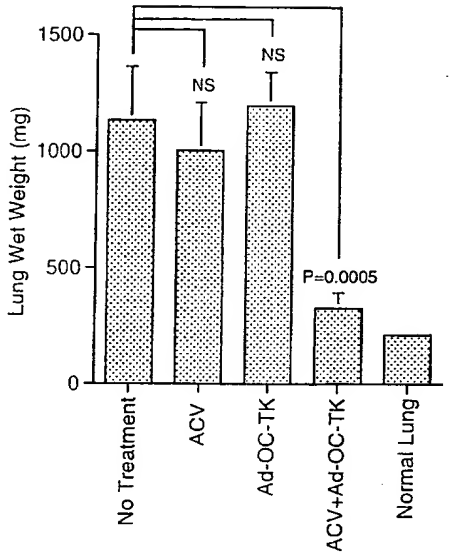

Figure 4. Reduction of the wet weight of lung-carrying osteosarcoma tumor nodules with Ad-OC-TK/ACV treatment. Animals bearing osteosarcoma lung metastases were treated with PBS, ACV, Ad-OC-TK, or Ad-OC-TK/ACV as described above. Animals were sacrificed 25 days after tumor cell inoculation, and lungs were removed for analysis. Animals receiving Ad-OC-TK/ACV treatment had significantly lighter lungs compared with those animals receiving other treatments ($P = .0005$, Student's t test). There were no significant differences in lung wet weight between animals receiving PBS, ACV, or Ad-OC-TK treatment.

centration of chemotherapeutic agents in the human lung by 10- to 20-fold.[23] This technique offers promise for the delivery of Ad-OC-TK and for the subsequent treatment of osteosarcoma lung metastasis through the systemic administration of ACV. The locoregional delivery of gene therapy can achieve higher local viral concentration and infectivity and can reduce viral leakage systemically. It is also expected that this route of

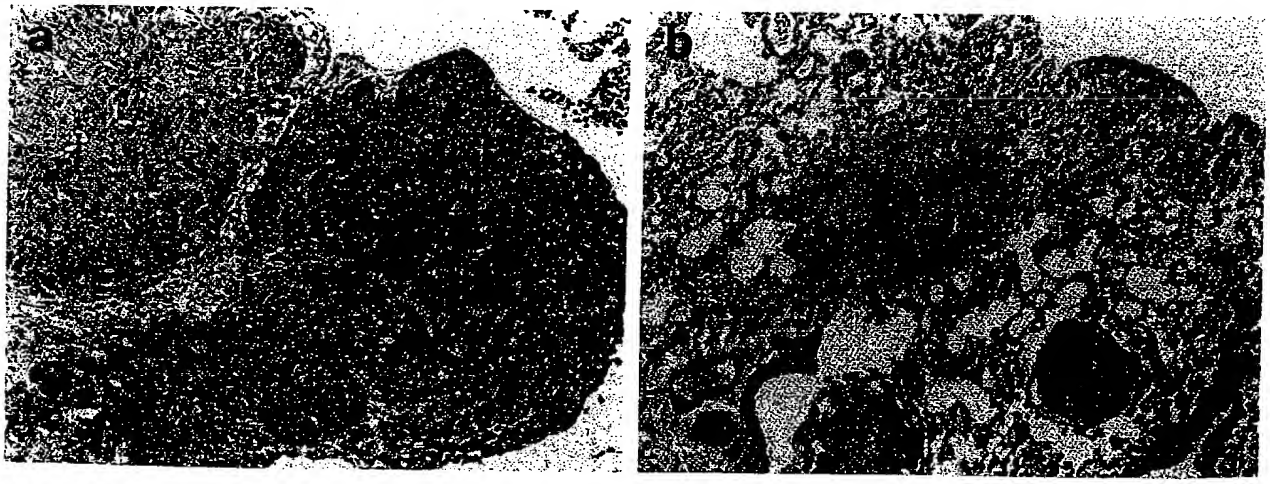

Figure 5. Tumor regression induced by Ad-OC-TK/ACV. Animals bearing osteosarcoma lung metastases were treated with PBS, ACV, Ad-OC-TK, or Ad-OC-TK/ACV as described in *Materials and Methods*. Animals were sacrificed 25 days after tumor cell inoculation, and lungs were removed for analysis. All nude mice receiving PBS, ACV, and Ad-OC-TK treatment had massive pulmonary metastatic tumor nodules (a) (with PBS), but Ad-OC-TK/ACV-treated animals had smaller tumor nodules (b) and extensive necrotic lesions (indicated by arrow) in the tumors. Tissue sections were photographed at low power.

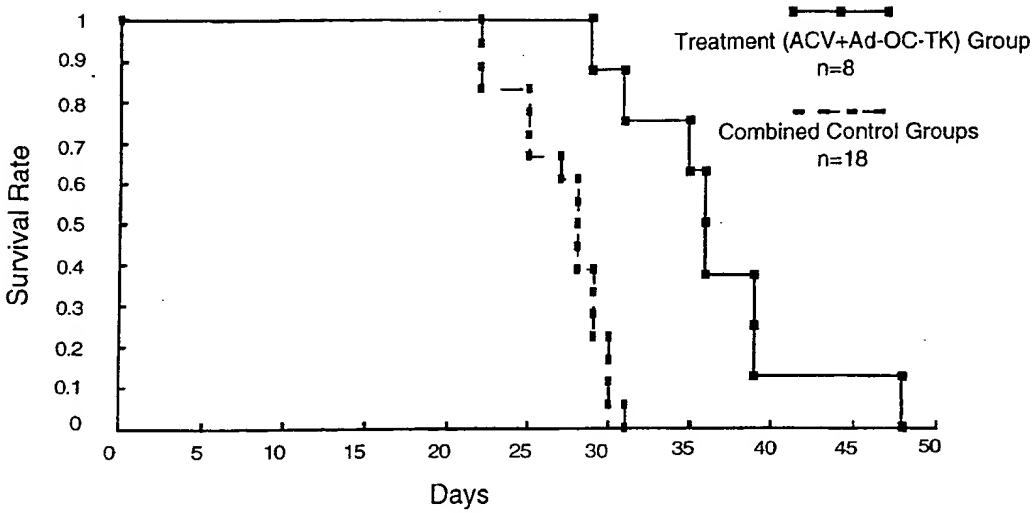

Figure 6. Survival of animals receiving Ad-OC-TK/ACV treatment. Animals bearing osteosarcoma lung metastases were treated with PBS (six animals), ACV (six animals), Ad-OC-TK (six animals), or Ad-OC-TK/ACV (eight animals) as described above. The survival study end-points were either animal death or sacrifice per a request by animal care personnel due to distress indicated by lethargy, ruffled fur, or weight loss. Since there were no significant differences between the three control groups, comprised of animals receiving PBS, ACV, or Ad-OC-TK, these data were combined as control animals in the Kaplan-Meier survival rate study. The survival rate of Ad-OC-TK/ACV-treated animals was significantly prolonged ($.005 < P < .01$, generalized Wilcoxon signed-rank test) when compared with the combined control animals.

gene therapy delivery will avoid the interference of neutralizing Abs, which can be flushed out of lung circulation prior to Ad administration. We are currently developing this locoregional technique for the treatment of osteosarcoma pulmonary metastasis using a larger size of animal model (eg, nude rat) in our laboratory.

In summary, we have shown for the first time that a rAd can be administered systemically to achieve a therapeutic effect on osteosarcoma lung metastasis. Ad-OC-TK/ACV dramatically inhibited the growth of lung nodules and significantly increased the survival of animals bearing osteosarcoma pulmonary metastases. This approach will open new avenues for targeting pulmonary metastasis using tissue-specific or tumor-specific promoters to guide the expression of therapeutic genes.

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